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TITLE: POLYNUCLEOTIDE VACCINE FORMULA IN PARTICULAR
AGAINST BOVINE RESPIRATORY PATHOLOGY

INVENTORS: Jean-Christophe AUDONNET, Annabelle BOUCHARDON, Philippe
BAUDU and Michel RIVIERE

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William S. Frommer
Registration No. 25,506
Thomas J. Kowalski
Registration No. 32,147
FROMMER, LAWRENCE & HAUG LLP
745 Fifth Avenue
New York, New York 10151
Tel: (212) 588-0800

**POLYNUCLEOTIDE VACCINE FORMULA IN PARTICULAR AGAINST
BOVINE RESPIRATORY PATHOLOGY**

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The present invention relates to a vaccine
5 formula allowing the vaccination of bovines in parti-
cular against respiratory pathology. It also relates to
a corresponding method of vaccination.

All bovines are carriers of viruses and
bacteria which are potentially pathogenic in widely
10 variable degrees.

Viruses can multiply when the specific immunity
is weakened and when there are lesions of the respira-
tory tract. They are then excreted by the animal and
may then contaminate other animals.

15 Among the viruses which are encountered, there
may be mentioned in particular the type 3 parainfluenza
virus (PI-3), of moderate inherent pathogenicity, the
bovine respiratory syncytial virus (RSV) and the bovine
herpesvirus (BHV) also called infectious bovine
20 rhinotracheitis (IBR) virus, of high inherent
pathogenicities.

Another virus which is particularly important
for its immunodepressant role and its harmful effects
on reproduction is the mucosal disease virus or bovine
25 pestivirus (BVDV).

These viruses generally manifest themselves by
a primary phase of hyperthermia, flu syndrome and
respiratory disorders, with digestive disorders
(diarrhoeas) in the case of BVD. This phase may be
30 accompanied by a secondary phase with the onset of
bronchopneumonia linked to bacterial, in particular
Pasteurella, infections which can lead to death. This
phenomenon is exacerbated in particular by the immuno-
depression resulting from BVD infection or by the
35 infection of macrophages by PI-3. Other symptoms may
further appear, such as abortions with BVD and BHV.

It therefore appears necessary to try to
develop an effective prevention against the principal
viruses involved in bovine respiratory pathology.

Associations of vaccines against certain viruses responsible for bovine respiratory pathology have already been proposed in the past.

5 The associations developed so far were prepared from inactivated vaccines or live vaccines and, optionally, mixtures of such vaccines. Their development poses problems of compatibility between valencies and of stability. It is indeed necessary to ensure both the compatibility between the different vaccine valencies, 10 whether from the point of view of the different antigens used or from the point of view of the formulations themselves, especially in the case where both inactivated vaccines and live vaccines are combined. The problem of the conservation of such combined vaccines and also of their safety especially in the presence of 15 an adjuvant also exists. These vaccines are in general quite expensive.

Patent Applications WO-A-90 11092, WO-A-93 19183, WO-A-94 21797 and WO-A-95 20660 have made use of the 20 recently developed technique of polynucleotide vaccines. It is known that these vaccines use a plasmid capable of expressing, in the host's cells, the antigen inserted into the plasmid. All the routes of administration have been proposed (intraperitoneal, 25 intravenous, intramuscular, transcutaneous, intradermal, mucosal and the like). Various vaccination means can also be used, such as DNA deposited at the surface of gold particles and projected so as to penetrate into the animal's skin (Tang et al., Nature 356, 30 152-154, 1992) and liquid jet injectors which make it possible to transfect at the same time the skin, the muscle, the fatty tissues and the mammary tissues (Furth et al., Analytical Biochemistry, 205, 365-368, 1992).

35 The polynucleotide vaccines may also use both naked DNAs and DNAs formulated, for example, inside cationic lipid liposomes.

G.J.M. COX has already proposed polynucleotide vaccination against type 1 bovine herpes virus in J. of

Virology, Volume 67, No. 9, September 1993, 5664-5667. The authors have also described plasmids integrating the gI (gB), gIII (gC) and gIV (gD) genes.

5 In Vaccine, Volume 13, No. 4, 415-421, 1995, J.E. CROWE presents a general review of the different methods of vaccination against respiratory syncytial virus and against type 3 parainfluenza virus. This review reexamines all the possibilities offered by the current vaccination techniques and simply suggests that
10 the polynucleotide immunization technique could be useful in the immunization strategy against RSV and PI-3. No plasmid construction or result of vaccination of bovines against these viruses is described in this document.

15 The invention therefore proposes to provide a multivalent vaccine formula which makes it possible to ensure vaccination against a number of pathogenic viruses involved in particular in bovine respiratory pathology and thus to ensure effective vaccination
20 against this pathology.

Another objective of the invention is to provide such a vaccine formula combining different valencies while exhibiting all the criteria required for mutual compatibility and stability of the
25 valencies.

Another objective of the invention is to provide such a vaccine formula which makes it possible to combine different valencies in the same vehicle.

30 Another objective of the invention is to provide such a vaccine formula which is easy and inexpensive to use.

Yet another objective of the invention is to provide such a vaccine formula and a method for vaccinating bovines which makes it possible to obtain a
35 multivalent protection with a high level of efficiency and of long duration, as well as good safety and an absence of residues.

The subject of the present invention is therefore a vaccine formula in particular against bovine

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respiratory pathology, comprising at least three polynucleotide vaccine valencies each comprising a plasmid integrating, so as to express it in vivo in the host cells, a gene with one bovine respiratory pathogen valency, these valencies being selected from the group consisting of bovine herpesvirus, bovine respiratory syncytial virus, mucosal disease virus and type 3 parainfluenza virus, the plasmids comprising, for each valency, one or more of the genes selected from the group consisting of gB and gD for the bovine herpesvirus, F and G for the bovine respiratory syncytial virus, E2, C + E1 + E2 and E1 + E2 for the mucosal disease virus, and HN and F for the type 3 parainfluenza virus.

Valency in the present invention is understood to mean at least one antigen providing protection against the virus for the pathogen considered, it being possible for the valency to contain, as subvalency, one or more modified or natural genes from one or more strains of the pathogen considered.

Pathogenic agent gene is understood to mean not only the complete gene but also the various nucleotide sequences, including fragments which retain the capacity to induce a protective response. The notion of a gene covers the nucleotide sequences equivalent to those described precisely in the examples, that is to say the sequences which are different but which encode the same protein. It also covers the nucleotide sequences of other strains of the pathogen considered, which provide cross-protection or a protection specific for a strain or for a strain group. It also covers the nucleotide sequences which have been modified in order to facilitate the in vivo expression by the host animal but encoding the same protein.

Preferably, the vaccine formula according to the invention comprises the four valences.

As regards the BHV valency, use is preferably made of the two genes encoding gB and gD, in different plasmids or in one and the same plasmid. Optionally,

but less preferably, either of these genes can be used.

For the RSV valency, use is preferably made of the two G and F genes integrated into two different plasmids or into one and the same plasmid. Optionally,
5 but less preferably, the F gene can be used alone.

For the BVD valency, use will preferably be made of a plasmid integrating the E2 gene. Optionally, but less preferably, a plasmid coding for E1 and E2 together or for the combination consisting of C, E1 and
10 E2 can be used.

For the PI-3 valency, use is preferably made of the combination of the two HN and F genes in two different plasmids or in one and the same plasmid. It is also possible to use only the HN gene.

A preferred vaccine formula according to the invention comprises and ensures the expression of the
15 BHV gB and gD genes, the RSV G and F genes, the BVD E2 genes and PI-3 HN and F genes.

The vaccine formula according to the invention
20 can be provided in a dose volume of between 0.1 and 10 ml and in particular between 1 and 5 ml.

The dose will be generally between 10 ng and 1 mg, preferably between 100 ng and 50 µg and preferably between 1 µg and 250 µg per plasmid type.

Use will preferably be made of naked plasmids simply placed in the vaccination vehicle which will be in general physiological saline (0.9% NaCl), ultrapure water, TE buffer and the like. All the polynucleotide vaccine forms described in the prior art can of course
25
30 be used.

Each plasmid comprises a promoter capable of ensuring the expression of the gene inserted, under its control, into the host cells. This will be in general a strong eukaryotic promoter and in particular a cytomegalovirus early CMV-IE promoter of human or murine origin,
35 or optionally of another origin such as rats, pigs and guinea pigs.

More generally, the promoter may be either of viral origin or of cellular origin. As viral promoter

other than CMV-IE, there may be mentioned the SV40 virus early or late promoter or the Rous sarcoma virus LTR promoter. It may also be a promoter from the virus from which the gene is derived, for example the gene's own promoter.

As cellular promoter, there may be mentioned the promoter of a cytoskeleton gene, such as for example the desmin promoter (Bolmont et al., Journal of Submicroscopic Cytology and Pathology, 1990, 22, 117-122; and Zhenlin et al., Gene, 1989, 78, 243-254), or alternatively the actin promoter.

When several genes are present in the same plasmid, these may be presented in the same transcription unit or in two different units.

The combination of the different vaccine valencies according to the invention may be preferably achieved by mixing the polynucleotide plasmids expressing the antigen(s) of each valency, but it is also possible to envisage causing antigens of several valencies to be expressed by the same plasmid.

The subject of the invention is also monovalent vaccine formulae comprising one or more plasmids encoding one or more genes from one of the viruses selected from the group consisting of BRSV, BVD and PI-3, the genes being those described above. Besides their monovalent character, these formulae may possess the characteristics stated above as regards the choice of the genes, their combinations, the composition of the plasmids, the dose volumes, the doses and the like.

The monovalent vaccine formulae may be used (i) for the preparation of a polyvalent vaccine formula as described above, (ii) individually against the actual pathology, (iii) combined with a vaccine of another type (live or inactivated whole, recombinant, subunit) against another pathology, or (iv) as booster for a vaccine as described below.

The subject of the present invention is in fact also the use of one or more plasmids according to the invention for the manufacture of a vaccine intended to

vaccinate bovines first vaccinated by means of a first conventional vaccine of the type in the prior art, in particular, selected from the group consisting of a live whole vaccine, an inactivated whole vaccine, a subunit vaccine, a recombinant vaccine, this first vaccine having, that is to say containing or capable of expressing, the antigen(s) encoded by the plasmid(s) or antigen(s) providing cross-protection.

Remarkably, the polynucleotide vaccine has a potent booster effect which results in an amplification of the immune response and the acquisition of a long-lasting immunity.

In general, the first-vaccination vaccines can be selected from commercial vaccines available from various veterinary vaccine producers.

The subject of the invention is also a vaccination kit grouping together a first-vaccination vaccine as described above and a vaccine formula according to the invention for the booster. It also relates to a vaccine formula according to the invention accompanied by a leaflet indicating the use of this formula as a booster for a first vaccination as described above.

The subject of the present invention is also a method for vaccinating bovines against respiratory pathology, comprising the administration of the effective vaccine formula as described above. This vaccination method comprises the administration of one or more doses of the vaccine formula, it being possible for these doses to be administered in succession over a short period of time and/or in succession at widely spaced intervals.

The vaccine formulae according to the invention can be administered, in the context of this method of vaccination, by the different routes of administration proposed in the prior art for polynucleotide vaccination and by means of known techniques of administration.

The subject of the invention is also the method of vaccination consisting in making a first vaccination

as described above and a booster with a vaccine formula according to the invention.

In a preferred embodiment of the process according to the invention, there is administered in a first instance, to the animal, an effective dose of the vaccine of the conventional, especially inactivated, live, attenuated or recombinant, type, or alternatively a subunit vaccine, so as to provide a first vaccination, and, within a period preferably of 2 to 6 weeks, the polyvalent or monovalent vaccine according to the invention is administered.

The invention also relates to the method of preparing the vaccine formulae, namely the preparation of the valencies and mixtures thereof, as evident from this description.

The invention will now be described in greater detail with the aid of the embodiments of the invention taken with reference to the accompanying drawings.

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- Figure No. 10 : Plasmid pAB071
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Sequence listing SEQ ID No.

- SEQ ID No. 1 : Sequence of the BHV-1 gB gene (strain ST)
- SEQ ID No. 2 : Oligonucleotide PB234
- SEQ ID No. 3 : Oligonucleotide PB235
- SEQ ID No. 4 : Oligonucleotide AB162
- SEQ ID No. 5 : Oligonucleotide AB163

SEQ ID No. 6 : Oligonucleotide AB026
SEQ ID No. 7 : Oligonucleotide AB027
SEQ ID No. 8 : Oligonucleotide AB028
SEQ ID No. 9 : Oligonucleotide AB029
5 SEQ ID No. 10 : Oligonucleotide AB110
SEQ ID No. 11 : Oligonucleotide AB111
SEQ ID No. 12 : Oligonucleotide AB114
SEQ ID No. 13 : Oligonucleotide AB115
SEQ ID No. 14 : Oligonucleotide AB116
10 SEQ ID No. 15 : Oligonucleotide AB117
SEQ ID No. 16 : Oligonucleotide AB130
SEQ ID No. 17 : Oligonucleotide AB131
SEQ ID No. 18 : Oligonucleotide AB132
SEQ ID No. 19 : Oligonucleotide AB133

EXAMPLES

Example 1: Culture of the viruses

The viruses are cultured on the appropriate
20 cellular system until a cytopathic effect is obtained.
The cellular systems to be used for each virus are well
known to persons skilled in the art. Briefly, the cells
sensitive to the virus used, which are cultured in
Eagle's minimum essential medium (MEM medium) or another
25 appropriate medium, are inoculated with the viral strain
studied using a multiplicity of infection of 1. The
infected cells are then incubated at 37°C for the time
necessary for the appearance of a complete cytopathic
effect (on average 36 hours).

Example 2: Extraction of the viral genomic DNAs

After culturing, the supernatant and the lysed
cells are harvested and the entire viral suspension is
centrifuged at 1000 g for 10 minutes at +4°C so as to
35 remove the cellular debris. The viral particles are then
harvested by ultracentrifugation at 400,000 g for 1 hour
at +4°C. The pellet is taken up in a minimum volume of
buffer (10 mM Tris, 1 mM EDTA). This concentrated viral
suspension is treated with proteinase K (100 µg/ml

final) in the presence of sodium dodecyl sulphate (SDS) (0.5% final) for 2 hours at 37°C. The viral DNA is then extracted with a phenol/chloroform mixture and then precipitated with 2 volumes of absolute ethanol. After
5 leaving overnight at -20°C, the DNA is centrifuged at 10,000 g for 15 minutes at +4°C. The DNA pellet is dried and then taken up in a minimum volume of sterile ultrapure water. It can then be digested with restriction enzymes.

10

Example 3: Isolation of the viral genomic RNAs

The RNA viruses were purified according to techniques well known to persons skilled in the art. The genomic viral RNA of each virus was then isolated using
15 the "guanidium thiocyanate/phenol-chloroform" extraction technique described by P. Chomczynski and N. Sacchi (Anal. Biochem., 1987, **162**, 156-159).

Example 4: Molecular biology techniques

20 All the constructions of plasmids were carried out using the standard molecular biology techniques described by J. Sambrook et al. (*Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989). All the
25 restriction fragments used for the present invention were isolated using the "Geneclean" kit (BIO 101 Inc. La Jolla, CA).

Example 5: RT-PCR technique

30 Specific oligonucleotides (comprising restriction sites at their 5' ends to facilitate the cloning of the amplified fragments) were synthesized such that they completely cover the coding regions of the genes which are to be amplified (see specific examples). The reverse
35 transcription (RT) reaction and the polymerase chain reaction (PCR) were carried out according to standard techniques (J. Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989). Each

RT-PCR reaction was performed with a pair of specific
amplimers and taking, as template, the viral genomic RNA
extracted. The complementary DNA amplified was extracted
with phenol/chloroform/isoamyl alcohol (25:24:1) before
5 being digested with restriction enzymes.

Example 6: plasmid pVR1012

The plasmid pVR1012 (Figure No. 1) was obtained
from Vical Inc., San Diego, CA, USA. Its construction
10 has been described in J. Hartikka et al. (Human Gene
Therapy, 1996, 7, 1205-1217).

**Example 7: Construction of the plasmid pPB156 (BHV-1 gB
gene)**

15 The BVH-1 bovine herpesvirus (ST strain)
genomic DNA (Leung-Tack P. et al. Virology, 1994, 199,
409-421) was prepared according to the technique
described in Example 2 and was digested with BamHI.
After purification, the 18 kbp BamHI-BamHI fragment was
20 cloned into the vector pBR322, previously digested with
BamHI, to give the plasmid pIBR-4-BamHI (22 kbp).

The plasmid pIBR-4-BamHI was then digested with
SalI in order to liberate a 6.6 kbp SalI-SalI fragment
containing the gene encoding the BHV-1 gB glycoprotein
25 (Figure No. 2 and SEQ ID No. 1). This fragment was
cloned into the vector pBR322, previously digested with
SalI, to give the plasmid pIBR-6,6-SalI (10.9 kbp).

The plasmid pIBR-6,6-SalI was digested with
NheI and BglII in order to liberate a 2676 bp NheI-
30 BglII fragment containing the gene encoding the bovine
herpesvirus (BHV-1) gB glycoprotein (fragment A).

A PCR reaction was carried out with the genomic
DNA from the bovine herpesvirus (BHV-1) (ST strain) and
with the following oligonucleotides:

35 PB234 (30 mer) (SEQ ID No. 2)
5'TTGTCGACATGGCCGCTCGCGGCGGTGCTC 3'

PB235 (21 mer) (SEQ ID No. 3)
5'GCAGGGCAGCGGCTAGCGCG 3'

so as to isolate the 5' part of the gene encoding the

BHV-1 gB glycoprotein. After purification, the 153 bp PCR product was digested with SalI and NheI in order to isolate a 145 bp SalI-NheI fragment (fragment B).

5 The fragments A and B were ligated together with the vector pVR1012 (Example 6), previously digested with SalI and BamHI, to give the plasmid pPB156 (7691 bp) (Figure No. 3).

10 **Example 8: Construction of the plasmid pAB087 (BHV-1 gD gene)**

A PCR reaction was carried out with the genomic DNA from the bovine herpesvirus (BHV-1) (ST strain) (P. Leung-Tack et al., Virology, 1994, **199**, 409-421), prepared according to the technique described in

15 Example 2, and with the following oligonucleotides:

AB162 (31 mer) (SEQ ID No. 4)

5'AAACTGCAGATGCAAGGGCCGACATTGGCCG 3'

AB163 (30 mer) (SEQ ID No. 5)

5'ATCTTGTTACCATATGACCGTGGCGTTG 3'

20 so as to amplify the 5' part of the gene encoding the bovine herpesvirus (BHV-1) gD glycoprotein (GenBank sequence accession No. = L26360) in the form of a 338 bp PCR fragment. After purification, this fragment was digested with PstI and NdeI in order to isolate a

25 317 bp PstI-NdeI fragment (fragment A).

The plasmid pBHV001 (P. Leung-Tack et al., Virology, 1994, **199**, 409-421) was digested with NdeI and StyI in order to liberate a 942 bp fragment containing the 3' part of the gene encoding the BHV-1

30 gD glycoprotein (fragment B).

The fragments A and B were ligated together with the vector pVR1012 (Example 6), previously digested with PstI and XbaI, to give the plasmid pAB087 (6134 bp) (Figure No. 4).

35

Example 9: Construction of the plasmid pAB011 (BRSV F gene)

An RT-PCR reaction according to the technique described in Example 5 was carried out with the genomic

RNA from the bovine respiratory syncytial virus (BRSV) (391-2 strain) (R. Lerch et al., J. Virology, 1991, **181**, 118-131), prepared as indicated in Example 3, and with the following oligonucleotides:

- 5 AB026 (33 mer) (SEQ ID No. 6)
5'AAAACTGCAGGGATGGCGGCAACAGCCATGAGG 3'
AB027 (31 mer) (SEQ ID No. 7)
5'CGCGGATCCTCATTACTAAAGGAAAGATTG 3'

so as to isolate the gene encoding the F fusion glycoprotein (BRSV F) in the form of a 1734 bp PCR fragment. After purification, this fragment was digested with PstI and BamHI in order to isolate a 1715 bp PstI-BamHI fragment. This fragment was ligated with the vector pVR1012 (Example 6), previously digested with PstI and BamHI, to give the plasmid pAB011 (6587 bp) (Figure No. 5).

Example 10: Construction of the plasmid pAB012 (BRSV G gene)

20 An RT-PCR reaction according to the technique described in Example 5 was carried out with the genomic RNA from the bovine respiratory syncytial virus (BRSV) (391-2 strain) (R. Lerch et al., J. Virology, 1991, **64**, 5559-5569) and with the following oligonucleotides:

- 25 AB028 (32 mer) (SEQ ID No. 8)
5'AAAACTGCAGATGTCCAACCATACCCATCATC 3'
AB029 (35 mer) (SEQ ID No. 9)
5'CGCGGATCCCTAGATCTGTGTAGTTGATTGATTTG 3'

so as to isolate the gene encoding the G protein (BRSV G) in the form of a 780 bp PCR fragment. After purification, this fragment was digested with PstI and BamHI in order to isolate a 763 bp PstI-BamHI fragment. This fragment was ligated with the vector pVR1012 (Example 6), previously digested with PstI and BamHI, to give the plasmid pAB012 (5634 bp) (Figure No. 6).

Example 11: Construction of the plasmid pAB058 (BVDV C gene)

An RT-PCR reaction according to the technique

described in Example 5 was carried out with the genomic RNA from the bovine viral diarrhoea virus (BVDV) (Osloss strain) (L. De Moerlooze et al., J. Gen. Virol., 1993, **74**, 1433-1438), prepared according to the technique described in Example 3, and with the following oligonucleotides:

AB110 (35 mer) (SEQ ID No. 10)

5'AAAACTGCAGATGTCCGACACAAAAGCAGAAGGGG 3'

AB111 (47 mer) (SEQ ID No. 11)

5'CGCGGATCCTCAATAAAAATCATTCCCCTGCGACTTGAAACAAAAC 3'

so as to amplify a 342 bp fragment containing the gene encoding the C capsid protein from the BVDV virus. After purification, the RT-PCR product was digested with PstI and BamHI to give a 324 bp PstI-BamHI fragment.

This fragment was ligated with the vector pVR1012 (Example 6), previously digested with PstI and BamHI, to give the plasmid pAB058 (5183 bp) (Figure No. 7).

Example 12: Construction of the plasmid pAB059 (BVDV E1 "gene")

An RT-PCR reaction according to the technique described in Example 5 was carried out with the genomic RNA from the bovine viral diarrhoea virus (BVDV) (Osloss strain) (L. De Moerlooze et al., J. Gen. Virol., 1993, **74**, 1433-1438) and with the following oligonucleotides:

AB114 (32 mer) (SEQ ID No. 12)

5'ACGCGTCGACATGAAGAACTAGAGAAAGCCC 3'

AB115 (33 mer) (SEQ ID No. 13)

5'GCGGGATCCTCAGCCGGGTTTGCAAACTGGGAG 3'

so as to isolate the sequence encoding the BVDV virus E1 protein in the form of a 1381 bp PCR fragment. After purification, this fragment was digested with SalI and BamHI to give a 1367 bp SalI-BamHI fragment.

This fragment was ligated with the vector pVR1012 (Example 6), previously digested with SalI and BamHI, to give the plasmid pAB059 (6236 bp) (Figure

No. 8).

Example 13: Construction of the plasmid pAB060 (BVDV E2 "gene")

An RT-PCR reaction according to the technique described in Example 5 was carried out with the genomic RNA from the bovine viral diarrhoea virus (BVDV) (Osloss strain) (L. De Moerlooze et al., J. Gen. Virol., 1993, **74**, 1433-1438) and with the following oligonucleotides:

10 AB116 (36 mer) (SEQ ID No. 14)
5'ACGCGTCGACATGACGACTACTGCATTCCTGGTATG 3'
AB116 (33 mer) (SEQ ID No. 15)
5'CGCGGATCCTCATTGACGTCCCGAGGTCATTTG 3'

so as to isolate the sequence encoding the BVDV virus E2 protein in the form of a 1252 bp PCR fragment. After purification, this fragment was digested with SalI and BamHI to give a 1238 bp SalI-BamHI fragment.

This fragment was ligated with the vector pVR1012 (Example 6), previously digested with SalI and BamHI, to give the plasmid pAB060 (6107 bp) (Figure No. 9).

Example 14: Construction of the plasmid pAB071 (BPIV HN gene)

25 An RT-PCR reaction according to the technique described in Example 5 was carried out with the genomic RNA from the type 3 bovine parainfluenza virus (PI3 = BPIV) and with the following oligonucleotides:

AB130 (33 mer) (SEQ ID No. 16)
30 5'TTTGTGCGACATGGAATATTGGAAACACACAAAC 3'
AB131 (33 mer) (SEQ ID No. 17)
5'TTTGATCCTTAGCTGCAGTTTTTCGGAAGTTC 3'

so as to isolate the gene encoding the BPIV HN glycoprotein (HN gene sequence deposited by H. Shibuta in 1987. GenBank sequence accession No. = Y00115) in the form of a 1737 bp PCR fragment. After purification, this fragment was digested with SalI and BamHI in order to isolate a 1725 bp SalI-BamHI fragment. This fragment was ligated with the vector pVR1012 (Example 6),

previously digested with SalI and BamHI, to give the plasmid pAB071 (6593 bp) (Figure No. 10).

Example 15: Construction of the plasmid pAB072 (BPIV F gene)

An RT-PCR reaction according to the technique described in Example 5 was carried out with the genomic RNA from the type 3 bovine parainfluenza virus (PI3 = BPIV) and with the following oligonucleotides:

AB132 (30 mer) (SEQ ID No. 18)

5' TTTGTCGACATGATCATCACAAACACAATC 3'

AB133 (30 mer) (SEQ ID No. 19)

5' TTTGGATCCTCATTGTCTACTTGT TAGTAC 3'

so as to isolate the gene encoding the BPIV F glycoprotein (F gene sequence deposited by H. Shibuta in 1987. GenBank sequence accession No. = Y00115) in the form of a 1641 bp PCR fragment. After purification, this fragment was digested with SalI and BamHI in order to isolate a 1629 bp SalI-BamHI fragment. This fragment was ligated with the vector pVR1012 (Example 6), previously digested with SalI and BamHI, to give the plasmid pAB072 (6497 bp) (Figure No. 11).

Example 16: Preparation and purification of the plasmids

For the preparation of the plasmids intended for the vaccination of animals, any technique may be used which makes it possible to obtain a suspension of purified plasmids predominantly in a supercoiled form. These techniques are well known to persons skilled in the art. There may be mentioned in particular the alkaline lysis technique followed by two successive ultracentrifugations on a caesium chloride gradient in the presence of ethidium bromide as described in J. Sambrook et al. (*Molecular Cloning: A Laboratory Manual*, 2nd edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989). Reference may also be made to Patent Applications PCT WO 95/21250 and PCT WO 96/02658, which describe methods for producing, on an

industrial scale, plasmids which can be used for vaccination. For the purposes of the manufacture of vaccines (see Example 17), the purified plasmids are resuspended so as to obtain solutions at a high concentration (> 2 mg/ml) which are compatible with storage. To do this the plasmids are resuspended either in ultrapure water or in TE buffer (10 mM Tris-HCl; 1 mM EDTA, pH 8.0).

10 **Example 17: Manufacture of the associated vaccines**

The various plasmids necessary for the manufacture of an associated vaccine are mixed starting with their concentrated solutions (Example 16). The mixtures are prepared such that the final concentration of each plasmid corresponds to the effective dose of each plasmid. The solutions which can be used to adjust the final concentration of the vaccine may be either a 0.9% NaCl solution, or PBS buffer.

Specific formulations such as liposomes or cationic lipids, may also be used for the manufacture of the vaccines.

Example 18: Vaccination of bovines

The bovines are vaccinated with doses of 100 μ g, 250 μ g or 500 μ g per plasmid. The injections are performed with a needle by the intramuscular route either at the level of the *gluteus* muscle, or at the level of the neck muscles. The vaccinal doses are administered in volumes of between 1 and 5 ml.